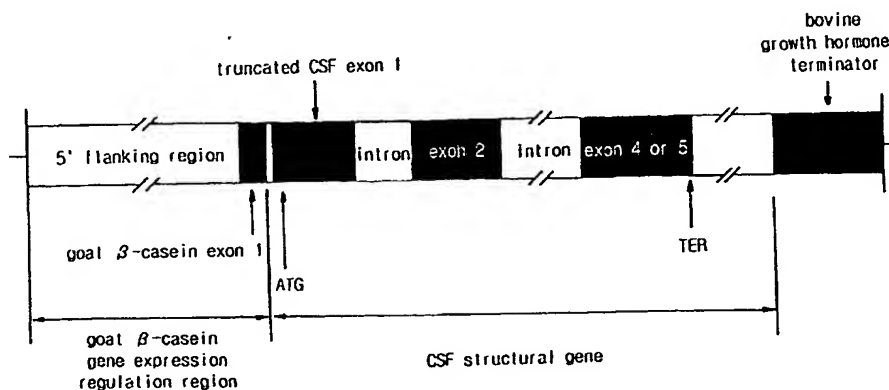




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(54) Title: **MAMMARY GLAND TISSUE-SPECIFIC EXPRESSION SYSTEM USING β -CASEIN PROMOTER SITE OF KOREAN NATIVE GOAT**



(57) Abstract

There are disclosed mammary gland tissue-specific expression systems using the promoter site for the β -casein gene of Korean native goats, by use of which physiological activating substances can be produced. In each of the expression systems, that is, novel plasmids pGbc, pGbc_L and pGbc_S (deposition Nos. KCTC 0515BP, 0514BP and 0513BP, respectively), a β -casein gene expression-regulating region, a physiological activating substance gene and a termination-regulating region are linked. Human granulocyte colony stimulating factor (hG-CSF) or human granulocyte macrophage colony stimulating factor (hGM-CSF) can be produced in HC11 cells, a mouse mammary gland tissue-derived cell line, and in the milk secreted from the transgenic mice by use of a hG-CSF or hGM-CSF gene-carrying pGbc, pGbc_L or pGbc_S in transfection into cell and microinjection to mouse. The proteins are those which experience the posttranslational modification and maintain their normal activity in the human body. The expression systems make it possible to easily produce the proteins at a great amount, to scale up protein production to the extent of industrialization, and to isolate and purify the desired protein with ease and safety.

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MAMMARY GLAND TISSUE-SPECIFIC EXPRESSION SYSTEM USING β -CASEIN PROMOTER SITE OF KOREAN NATIVE GOAT

Technical Field

5 The present invention relates to a mammary gland tissue-specific expression system using the promotor site for the β -casein gene of Korean native goats, through which physiological activating substances can be produced. More particularly, the present invention relates to novel
10 recombinant mammalian expression vectors in which a β -casein gene expression-regulating region, a physiological activating substance gene and a termination-regulating region are linked. Also, the present invention is concerned with a method for producing physiological activating substances in mammary gland tissue-derived cell lines and in animals, using the novel recombinant vectors.

15 Background Art

Physiological activating substances are produced and secreted at trace amounts in the human body and play an essential role in various metabolisms and modulations. The physiological activating substances known to date include insulin, interleukins, hemopoietic growth regulating
20 factors, such as stem cell factor, granulocyte colony stimulating factor, erythropoietin, etc, and are too numerous to describe their great functions in the human body, in detail. The reason why such physiological activating substances, in spite of their importance, have not yet been industrialized, is that they are difficult to isolate and purify owing to their
25 trace amount in the human body. Further, the physiological activating substances produced by using a procaryotic expression system, such as that obtained from E.coli, frequently do not perform their normal functions in the human body as well as have not yet overcome the safety problem which must be solved before administration.

30 According to the reports contributed to the academic circles, it is known that, even if there is used a promotor site for a gene which is expressed specifically in a mammary gland tissue, the expression level is different depending on the species from which the promotor is obtained and on the genes to be expressed (Clark et al. (1987) *Trends Biotech.* 5,

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20-24; Simons et al., (1987) *Nature* 328, 530-532; Lee et al., (1988) *Nucl. Acids Res.* 16, 1027-1041; Vilotte et al., (1988) *Eur. J. Biochem.* 186, 43-48; Gorden et al., (1987) *Bio/Technology* 8, 443-446; Shani et al., (1992) *Transgenic Res.* 1, 195-208; Wright et al., (1991) *Bio/Technology* 2, 830-834; Ebert et al., (1991) *Bio/Technology* 2, 835-838; Mega et al., (1994) *Transgenic Res.* 3, 36-42; Wei et al., (1995) *Transgenic Res.* 4, 232-240; Gutierrez et al., (1996) *Transgenic Res.* 5 271-279)

In order to produce physiological activating substances, the expression systems which take advantage of *E. coli* (Korean Pat. Publication No. 94-5585) and animal cells have been usually used. These techniques would occasionally bring about industrial successes, but still have significant problems to be solved. For instance, in the case of the expression utilizing *E. coli*, mass production is possible with low cost. However, since *E. coli*, a prokaryote, does not perform a posttranslational modification, which is a feature of eukaryotes, such a human physiological activating substance as EPO cannot exert its activity if it is produced in *E. coli*. To avoid this problem, active research has been and continues to be directed to the development expression systems which take advantage of animal cells. The products expressed in these systems are active in the human body because they experience posttranslational modifications. However, the problem of high cost for culturing animal cells remains unsolved.

Almost all of the physiological activating substances that are industrially produced utilize the above mentioned techniques, so they have the problems to be solved, including activity sustenance, cost, and isolation and purification.

Disclosure of the Invention

The mammary gland-specific expression systems developed by the present inventors, named pGbc, pGbc_L and pGbc_S, deposited in the Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology, on August 17, 1998 (deposition Nos. KCTC 0515BP, 0514BP and 0513BP, respectively) make it possible that desired proteins are produced by expression in mammary gland tissue-derived animal cells or through the milk secreted from the transgenic animals with the expression systems, thereby solving the above-mentioned

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problems, that is, the activity sustenance, production cost, and isolation and purification of the desired proteins.

5 Use of the expression-regulating region of a β -casein gene, expressed specifically in mammary gland tissues, in producing human physiological activating substances, brings about the following industrial advantages. First, because the target proteins which are produced by the recombination technique of the present invention experience the same posttranslational modification as that which the corresponding naturally occurring proteins do, the target proteins can sustain their activity in the human body. Secondly, by virtue of taking advantage of specificity for mammary gland tissue, the expression systems of the present invention employing mammary gland tissue-derived cells or transgenic animals can produce physiological activating substances at much lower cost than do the expression systems using general animal cells. The proteins produced in mammary gland tissue-derived cells or through the milk secreted from transgenic animals are few in number, so that the target protein is easy to isolate and purify. Additionally, transgenic animals require no further significant cost in scaling-up the production of the target proteins as well as produce no pollution during its production. A third advantage is the safety of the physiological activating substances produced. Because there are no toxins in the products secreted from the mammary gland tissues, the expression system of the present invention is safer than other conventional systems.

25 In order to producing physiological activating substances, methods utilizing E.coli, or animal cells were developed and more recently, advantage has been taken of transgenic animals. The expression techniques using E.coli as a host or using animal cells have now a limitation in industrial application owing to the above-mentioned problems, that is, the activity sustenance, production cost, and isolation and purification of the physiological activating substances produced. As a measure of settling these problems, transgenic animal and related techniques have rapidly developed and now make a great advance in biological studies.

35 The present invention uses a mammary gland tissue-derived cell line and a transgenic animal in producing proteins. For this, molecular biological technology and other apical techniques are employed in the present invention. For example, DNA recombination techniques are

needed for constructing the mammalian expression vectors which are able to be expressed specifically in mammary gland tissues and a microinjection technique is for producing a transgenic animal with the vectors.

5 Although it is well known to those skilled in the art to use the promoter sites of the genes expressed specifically in mammary gland tissues in constructing a mammalian expression vector which is able to express proteins specifically in the mammary gland tissues and the mammalian expression vector of the present invention is originated from
10 pRC/RSV, a commercial vector (Invitrogen Inc.), the expression systems of the present invention is quite different from those of other conventional techniques in the following aspects. First, the goat β -casein promoter used in the present invention is obtained from Korean native goats. A
15 second characteristically different point is that the goat β -casein promoter site is linked to the first exon of a structural gene via the first exon of the goat β -casein gene. In most cases, an intron is interposed between a promoter site and the first exon of a structural gene. Third, in the mammalian expression vectors according to the present invention, a bovine growth hormone follows a structural gene, with the aim of
20 accomplishing a preferable transcription termination. Irrespective of whether the poly A signal of the structural gene is present or not, the bovine growth hormone terminator is linked to the structural gene.

Brief Description of the Drawings

25 The above and other objects and aspects of the invention will become apparent from the following description of embodiments with reference to the accompanying drawings in which:

Fig. 1 a schematic view showing a portion of the common structure of novel vectors pGbc_S and pGbc_L for transfection into animal cells and a novel vector pGbc for transgenic animals;

30 Fig. 2 is the base sequence of the β -casein promoter of Korean native goats;

Fig. 3 is a schematic diagram illustrating the construction of the recombinant vector pGbc_S, according to the present invention;

35 Fig. 4 is a schematic diagram illustrating the construction of the recombinant vector pGbc_L, according to the present invention;

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Fig. 5 is a schematic diagram illustrating the recombination of the vector pGbc_S with a hGCSF gene, according to the present invention;

Fig. 6 is a schematic diagram illustrating the recombination of the vector pGbc_S with a hGMCSF gene, according to the present invention;

5 Fig. 7 is a schematic diagram illustrating the recombination of the vector pGbc_L with a hGCSF gene, according to the present invention;

Fig. 8 is a schematic diagram illustrating the recombination of the vector pGbc_L with a hGMCSF gene, according to the present invention

10 Fig. 9 is a schematic diagram illustrating the recombination of the vector pGbc with a hGCSF gene or a hGCSF gene, according to the present invention;

Fig. 10 shows a Western blotting analysis for the hGM-CSF proteins produced from the mouse mammary gland tissue-derived HC11 cells which are transfected with a recombinant vector pGbc_L or pGbc_S carrying a hGM-CSF gene, according to the present invention;

15 Fig. 11 is an ELISA graph for the hGM-CSF proteins produced from the mouse mammary gland tissue-derived HC11 cells which are transfected with a recombinant vector pGbc_L or pGbc_S carrying a hGM-CSF gene, according to the present invention;

20 Fig. 12 is an ELISA graph for the hG-CSF protein produced from the mouse mammary gland tissue-derived HC11 cells which are transfected with a recombinant vector pGbc_L or pGbc_S carrying a hG-CSF gene, according to the present invention;

25 Fig. 13 is a photograph of the PCR products obtained by using the genomic DNAs of transgenic mice as templates;

Fig. 14 shows a Western blotting analysis for the hG-CSF proteins secreted in the milk from the transgenic mice, according to the present invention;

30 Fig. 15 is an ELISA graph for the hG-CSF proteins secreted in the milk from the transgenic mice, according to the present invention; and

Fig. 16 is an ELISA graph for the hGM-CSF proteins secreted in the milk from the transgenic mice, according to the present invention.

Best Modes for Carrying Out the Invention

[1] Construction of Expression Vectors

1) Amplification of the B-casein Promotor and Exons 1 and 2 of Korean Native Goats

Two pairs of primers are designed to amplify through a polymerase chain reaction (PCR) a portion of the β -casein gene which was reported to be expressed specifically in Korean native goats. One pair of the primers is responsible for the amplification of a partial gene including the goat β -casein promoter site and exon 1 and the other for the amplification of a partial gene including the goat β -casein intron 1 and exon 2. The PCR product for the goat β -casein promoter and exon 1 is digested with endonucleases Sac I and Hind III while the PCR product for the goat β -casein intron 1 and exon 2 with endonucleases Hind III and Sal I. The two upstream and downstream primers for the amplification of the goat β -casein and exon 1 are named GBC-F1 and GBC-R1, respectively and have the following base sequences: GBC-F1, 5'-GCT GAG CTC TTT AGT ATA TTG TTA AGG A-3'; and GBC-R1, 5'-TGT CAA GCT TAT CTT AAA CAC CCT TA-3'. The two upstream and downstream primers for the amplification of the goat β casein intron 1 and exon 2 are named GBC-F2 and GBC-R2, respectively, and have the following base sequences: GBC-F2, 5'-GCA TAA GCT TTA CAC TAT TTT CAG CAG-3'; and GBC-R2, 5'-ATA GTC GAC CCA GAG TTG TGG TC-3'. After the two digested PCR products together are inserted in a pBluescript II SK vector, commercially available from Stratagene, the resulting plasmid is subjected to double strand sequencing analysis to identify the base sequence of the ligated gene fragments.

2) Construction of PGbc_S and pGbc_L

Of the gene region subcloned in the pBluescript II SK (Stratagene), a stretch of DNA ranging from 501 nucleotides to one nucleotide on the upstream side of the translation start codon for exon 1 is amplified by PCR. This PCR product is digested with restriction enzymes Nru I and Hind III. Separately, pRC/RSV, a mammalian expression vector, is treated with the same enzymes to remove the LTR region. To this opened plasmid, the digested PCR product is inserted by ligation to give pGbc_S.

The digested PCR product is further treated with restriction enzyme Dra I and extraction and purification are made for the gene fragment whose opposite ends are cut by Hind III and Dra I. The pBluescript II SK comprising the goat β -casein promoter, exon 1, intron 1 and exon 2 regions is subjected to double digestion with restriction enzymes Stu I and

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Dra I, followed by extraction and purification for the fragment comprising no exon 1 gene. These two gene fragments together are ligated to a pRc/RSV vector which is previously cleaved by Nru I and Hind III, to give pGbc_L.

- 5 Whether pGbc_S and pGbc_L are correctly constructed or not is confirmed by base sequencing analysis.

- 3) Construction of pGbc and Recombination with Physiological activating substance Genes (human Granulocyte Colony Stimulating Factor (hGCSF) and human Granulocyte Macrophage Colony Stimulating Factor (hGMCSF))
- 10

The PCR product A gene fragment including the promoter site and exon 1 region is double-digested with Sac I and Dra I to give a gene fragment including the promoter site and a partial exon 1 extending to one nucleotide on the 5' side of the translation start codon. Separately, the PCR product 500 bp in size obtained in 2) above, is digested with Dra I and Hind III. By ligation, these two gene fragments are together inserted in a Sac I/Hind III-cleaved pBluescript II SK vector (Stratagene) which is, then, opened by the cleavage with Hind III and EcoR I. To this opened plasmid, a truncated gene fragment which includes a structural gene linked to a bovine growth hormone terminator and which is truncated with Hind III and EcoR I, is ligated. The general structure of the pGbc type vectors is schematically shown in Fig. 1.

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[2] Recombination of Mammary Gland Tissue-Specific Expression Vectors & hGCSF Gene and hGMCSF Gene

- 25 1) Using well-known gene search and PCR techniques (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, New York), an hGCSF gene and an hGMCSF gene are cloned in pUC19, a plasmid vector commercially available.

- 2) Recombination of hGCSF Gene and Mammary Gland Tissue-Specific Expression Vectors pGbc_S and pGbc_L
- 30

The pUC19 vector in which the hGCSF gene is cloned is digested with restriction enzymes BamH I and Xba I. Fragment 1, which includes a region ranging from exon 2 through a poly A signal, is isolated and purified. The vector thus opened is digested with restriction enzyme Pst I to give Fragment 2 which includes a region ranging from one nucleotide

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on the 5' side of the translation start codon through one nucleotide on the 5' side of the exon 2. Fragments 1 and 2 are inserted and ligated to a pBluescript II SK vector which is opened with restriction enzymes Pst I and Xba I, to produce a recombinant plasmid pBluescript II SK-hGCSF. This plasmid is subjected to double digestion with endonucleases Hind III and Xba I to obtain a modified hGCSF gene which is, then, ligated to a pGbc_S vector which is digested at the same endonuclease sites Hind III and Xba I, to make a novel plasmid pGbc_S-hGCSF.

Similarly, a novel plasmid pGbc_L-hGCSF is constructed by ligating a fragment which is obtained by the endonuclease digestion of a goat β -casein promoter and a modified hGCSF gene fragment which is obtained by double digestion of the pBluescript II SK-hGCSF with Hind III and Xba I to a pGbc_L vector opened with the same endonucleases.

3) Recombination of hGMCSF Gene and Mammary Gland-Specific Expression Vectors pGbc_S and pGbc_L

The hGMCSF gene which is subcloned in the pUC19 vector is extracted by digestion with restriction enzymes BamH I and EcoR I and then, inserted by ligation to a pBluescript II SK vector (Stratagene) which is digested with the same endonucleases, to produce a recombinant plasmid pBluescript II SK-hGMCSF. A modified gene is recovered by the digestion of the recombinant plasmid with Hind III and Xba I and then, ligated to a pGbc_S vector which is digested at the same endonuclease sites Hind III and Xba I, to construct a novel plasmid pGbc_S-hGMCSF.

Similarly, a novel plasmid pGbc_L-hGMCSF is constructed by ligating a fragment which is obtained by the endonuclease digestion of a goat β -casein promoter and a modified hGMCSF gene fragment which is obtained by double digestion of the pBluescript II SK-hGMCSF with Hind III and Xba I to a pGbc_L vector opened with the same endonucleases.

The successful construction of the expression vectors is confirmed through base sequencing analysis.

[3] Transfection of pGbc_S-hGCSF/hGMCSF and pGbc_L-hGCSF/hGMCSF into Mouse Mammary Gland Tissue-derived HC11 cell and Expression Induction with Lactating Hormone

1) Culture of HC11 Cell Strain

HC11 cells are cultured in RPMI media, commercially available from Gibco BRL, supplemented with fetal bovine serum at a final concentration of 10 %, epidermal growth factor at 10 ng/ml, insulin at 5 μ g/ml and gentamicin (Sigma) at 50 μ g/ml.

2) Transfection of pGbc_S-hGCSF, pGbc_S-hGMCSF, pGbc_L-hGCSF and pGbc_L-hGMCSF into HC11 Cells

The four novel plasmids constructed in the present invention are purified by using QIAGEN-tip 100, commercially available from Qiagen company, according to the protocol recommended by Qiagen company. The introduction of the purified plasmids into HC11 cells is performed using an electroporator, manufactured by Invitrogen. A detailed procedure follows the protocol recommended by the provider.

3) Selection with Antibiotics

The HC11 cells are transferred to T25 flasks and cultured for 24-48 hours in an incubator which is maintained at 5% CO₂ and at 37 °C, after which the culture media are changed with fresh RPMI 1640 media (Gibco BRL), supplemented with fetal bovine serum at a final concentration of 10%, epidermal growth factor at 10 ng/ml, insulin at 5 μ g/ml, and antibiotics gentamicin (Sigma) at 50 μ g/ml and geneticin (Sigma) at 200 μ g/ml, to select the transfected cells.

4) Expression Induction with Lactating Hormone

After the selection, the selective media are changed with induction media comprising RPMI 1640 media (Gibco BRL) supplemented with insulin at a final concentration of 5 μ g/ml, geneticin (Sigma) at 200 μ g/ml, gentamicin 50 μ g/ml, goat prolactin at 5 μ g/ml and dexamethasone at 1 μ m. The cells are cultured in a 5% CO₂, 37 °C incubator for 4 days without refreshing the media.

5) Expression Level Assay

The human physiological activating substances produced as a result of the expression induction of their genes are secreted into the media. A Western blotting technique is used for the qualitative analysis of the secreted products while an enzyme linked immunosorbent assay (ELISA) is for a quantitative analysis. As a primary antibody for the Western blotting, anti-human G-CSF mouse Ig G monoclonal or polyclonal antibody is used for the analysis of the expression of human granulocyte colony stimulating factor and anti-human GM-CSF mouse Ig G for the

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analysis of the expression of human granulocyte macrophage colony stimulating factor. Horse radish peroxidase-conjugated anti-mouse Ig G is used as a secondary antibody for the Western blotting. For ELISA, anti-human G-CSF goat Ig G polyclonal antibodies or anti-human GM-CSF goat Ig G polyclonal antibodies are first attached on 96 well plates which are, then, treated with the expressed product as a corresponding antigen or with a commercially available factor used as a standard. To these were linked the anti-human G-CSF or anti-human GM-CSF monoclonal antibody which is the same as used in the Western blotting. The resulting antibody complexes are treated with alkali phosphatase-conjugated anti-mouse Ig G monoclonal antibody with the aim of inducing a coloring reaction (Ed Harlow and Davis Lane (1989) *Antibodies A Laboratory Manual*, Cold Spring harbor Laboratory Press, New York).

15 [4] Expression in Transgenic Mouse

1) Purification of the Vectors for Transfection

For purifying the vectors for transfection from E. coli, QIAGEN tip 100 (Qiagen) and Elutip (Schleicher & Schuell) are used. First, the vectors for transfection are purified with the aid of QIAGEN tip 100. The purified vectors are digested with restriction enzymes, followed by extraction with a GeneClean II kit. The extracted vectors are further purified by following the protocol recommended by Schleicher & Schuell and then, dialyzed in a solution comprising 10 mM Tris-Cl (pH 7.2) and 10 mM EDTA, to produce a vector at an amount of 40 ng/ml, which is later used for microinjection.

2) Microinjection

Into the male pronuclear site of a CBA line mouse oosperm, the finally purified expression vector is introduced by microinjection. This oosperm is nidated in the womb of a surrogate mother by using a surgical operation technique.

3) Genomic DNA Isolation and Gene Induction Assay

The progenies from the surrogate mother have their tails cut. From them, genomic DNA is isolated and purified according to a known method (Vilotte, J. -L. et al., (1989) *Eur. J. Biochem.* 186, 43-48). Whether the desired gene is correctly introduced into the mice or not is

identified by appropriate methods including Southern blotting and PCR.

4) Milk Extraction and Expression Level Assay

The progeny mice into which the genes are introduced correctly are let to copulate non-transgenic, normal mice to produce next generation progenies. 10 days after birth, the transgenic parturient mice are segregated from the their offspring for 3 hours. Following peritoneal injection of oxytocin, milk is extracted from the parturient mice. The expression level of the genes is assayed in the same manner as in cell level, using Western blotting and ELISA for qualitative and quantitative analyses, respectively.

Now, a further description will be given of the expression system according to the present invention, with reference to the drawings.

Referring to Fig. 1, there is a gene structure showing recombinant vectors pGbc_S, pGbc_L and pGbc. The first two vectors are constructed for the expression in animal cells while the last is for transgenic mouse. As shown in Fig. 1, this gene structure comprises a goat β -casein gene expression regulation region, a CSF structural gene region and a bovine growth hormone terminator region.

The β -casein gene expression regulation region consists of a partial β -casein exon 1 which extends only to one nucleotide on the 5' side of the translation start codon, and a 5' flanking region including goat β -casein promoter site.

In the CSF structural gene region, "ATG", the start codon, is written to emphasize that it comes from the physiological activating substance to be expressed, itself. The hG-CSF gene consists of 4 exons whereas the hGM-CSF gene of 5 exons, for which "exon 4 or 5" stands. "TER" denotes the termination codon for the CSF gene.

In this figure, the distances of the regions are not proportional to their actual lengths.

Fig. 2 is the base sequence of the β -casein promoter from the Korean native goat utilized in the present invention. The base sequence is identical to that of *Capra hircus* β -casein promoter and exon 1, reported by Roberts, B.T., Ditullio, P., Vitale, J., Hehir, K., and Gordon, K. in *Gene* (1992) 121, 255-262.

With reference to Fig. 3, there is schematically shown a construction procedure for the recombinant vector pGbc_S. In the figure, the length of each region does not reflect the scale of its actual length.

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That two lines join together into one arrow line illustrates ligation while MCS stands for the multicloning site, PCR product is the product obtained by a polymerase chain reaction, and RSV LTR represents Rous sarcoma virus long tandem repeat. The thicker lines in the PCR product and pGbc_S illustrations denote the exon 1 of a goat β -casein gene.
5 Restriction enzymes are positioned over their own recognition sites on the illustrations.

With reference to Fig. 4, there is schematically shown a construction procedure for the recombinant vector pGbc_L. In the figure,
10 the length of each region does not reflect the scale of its actual length. One arrow line into which three lines join together stands for ligation, MCS stands for the multicloning site, PCR product is the product obtained by a polymerase chain reaction, and RSV LTR is Rous sarcoma virus long tandem repeat. The thicker lines in the PCR product, goat β -
15 casein promoter & exon 1 and pGbc_L illustrations denote the exon 1 of a goat β -casein gene.

With reference to Fig. 5, there is schematically shown a procedure for the recombination between the plasmid pGbc_S and the hGCSF gene.

In this figure, the lengths of plasmids and DNA fragments are only
20 illustrative, but do not reflect the scale of their actual lengths. Restriction enzymes are positioned over their own recognition sites on the illustrations. Ligation is expressed by joining two or more lines together into one arrow line. Exon genes are expressed by thicker lines than those expressing intron genes. In pBluescript II SK-hGCSF, pGbc_S and
25 pGbc_S-hGCSF illustrations, exons and introns are indiscriminately expressed.

With reference to Fig. 6, there is schematically shown a procedure for the recombination between the plasmid pGbc_S and the hGMCSF gene.

In this figure, the lengths of plasmids and DNA fragments are only
30 illustrative but do not reflect the scale of their actual lengths. Restriction enzymes are positioned over their own recognition sites on the illustrations. Ligation is expressed by joining two or more lines together into one arrow line. Exon genes are expressed by thicker lines than those
35 expressing intron genes. In pBluescript II SK-hGMCSF, pGbc_S and pGbc_S-hGMCSF illustrations, exons and introns are indiscriminately expressed.

Fig. 7 is a schematic diagram showing a procedure for the recombination between the plasmid pGbc_L and the hGCSF gene.

In this figure, the length of each of the plasmids and DNA fragments does not reflect the scale of its actual length. Restriction enzymes are positioned over their own recognition sites on the illustrations. The one arrow line into which many lines join together illustrates ligation. In the illustration for the DNA fragment obtained by the digestion of the pBluescript II SK-hGCSF vector with restriction enzymes Hind III and Xba I, thicker lines stand for exons. In the other illustrations, exons and introns are indiscriminately expressed.

Fig. 8 is a schematic diagram showing a procedure for the recombination between the plasmid pGbc_L and the hGMCSF gene.

In this figure, the length of each of the plasmids and DNA fragments does not reflect the scale of its actual length. Restriction enzymes are positioned over their own recognition sites on the illustrations. The one arrow line into which many lines join together illustrates ligation. In the illustration for the DNA fragment obtained by the digestion of the pBluescript II SK-hGMCSF vector with restriction enzymes Hind III and Xba I, thicker lines stand for exons. In the other illustrations, exons and introns are indiscriminately expressed.

Fig. 9 shows a recombination of the pGbc vectors with hGCSF and hGMCSF genes.

In this figure, the length of each of the plasmids and DNA fragments does not reflect the scale of its actual length. Restriction enzymes are positioned over their own recognition sites on the illustrations. The one arrow line into which many lines join together illustrates ligation. In the illustrations for goat β -casein promoter & exon 1 and PCR product, thicker lines stand for exons.

Referring to Fig. 10, there is shown a qualitative analysis for the expression of hGM-CSF in HC11 cells by a Western blotting technique. Proteins for this Western blotting are obtained from the HC11 cells, a cell line derived from the mouse mammary gland tissue, which are transfected with each of the mammalian expression vectors pGbc_S and pGbc_L, each containing an hGM-CSF gene, by inducing the cells into expression.

Referring to Fig. 11, there is shown a quantitative analysis for the expression of hGM-CSF in HC11 cells by ELISA. Proteins for ELISA are obtained from the HC11 cells transfected with each of the mammalian

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expression vectors pGbc_S and pGbc_L, in which an hGM-CSF gene is cloned, by inducing the cells into expression.

Referring to Fig. 12, there is shown a quantitative analysis for the expression of hG-CSF in HC11 cells by ELISA. Proteins for ELISA are obtained from the HC11 cells transfected with each of the mammalian expression vectors pGbc_S and pGbc_L, in which an hG-CSF gene is cloned, by inducing the cells into expression.

Fig. 13 is a photograph showing the electrophoresis of PCR products of hGCSF genes on an agarose gel. In the figure, Lane (+) denotes an hGCSF PCR product using as a template the plasmid pGbc-hGCSF. A PCR product, using as a template the genomic DNA of the transgenic mice which are born from a surrogate mouse in whose womb an oosperm transfected with the plasmid pGbc-hGCSF is nidated, is electrophoresed on Lane TG-2. TG-2.1 stands for the transgenic progenies from TG-2. On Lane (-) is run a PCR product which uses as a template the genomic DNA of non-transgenic, that is, normal mice.

Referring to Fig. 14, there is shown a qualitative analysis for the expression of hG-CSF in the milk secreted from transgenic mice by a Western blotting technique. Proteins for this Western blotting are obtained from the milk secreted from the transgenic mice into which the mammalian expression vectors pGbc_S and pGbc_L, each containing an hG-CSF gene, are introduced.

Referring to Fig. 15, there is shown a quantitative analysis for the expression of hG-CSF in the milk secreted from transgenic mice by ELISA. Proteins for ELISA are obtained from the milk secreted from the transgenic mice into which the mammalian expression vectors pGbc_S-hGCSF and pGbc_L-hGCSF are introduced.

Referring to Fig. 16, there is shown a quantitative analysis for the expression of hG-CSF in the milk secreted from transgenic mice by ELISA. Proteins for ELISA are obtained from the milk secreted from the transgenic mice into which the mammalian expression vectors pGbc_S-hGMCSF and pGbc_L-hGMCSF are introduced.

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE I: Construction of the Expression Vectors for HC11 Cell

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Using Goat β -Casein Promoter site and Bovine Growth Hormone Terminator.

1) Construction of pGbc_S Vector

From a vector pBluescript II SK (Stratagene) in which a DNA
5 fragment including the promoter site, exon 1, intron 1 and exon 2 of a
goat β casein gene was subcloned, a stretch of DNA which covered from
501 nucleotides to one nucleotide on the 5' side of the translation start
codon for exon 1, was obtained by a PCR using primers CAS-F1 and
CAS-R1, which had a base sequence of 5'-TGA TCG, CGA GTC CAC
10 CAG GCT CTA CTG TC-3' and 5'-GAG AAG CTT AAT GGA TAA
TGA TCT GA-3', respectively. The PCR consisted of 35 thermal cycles
in which heating was performed in the order of at 94 °C for 3 min, at 55
°C for 1 min and at 72 °C for 1 min for the first cycle, in the order of at
94 °C for 1 min, at 55 °C for 1 min and at 72 °C for 1 min for cycles 2-
15 34, and in the order of at 94 °C for 1 min, at 55 °C for 1 min and at 72
°C for 5 min for the last cycle.

In a mammalian expression vector pRc/RSV which was digested
with endonucleases Nru I and Hind III to open it and remove its LTR, the
PCR product, after being also double-digested with the same
20 endonucleases, was inserted by ligation. In result, a novel plasmid
pGbc_S was obtained, as summarized in Fig. 3.

2) Construction of pGbc_L Vector

The truncated PCR product of the above 1) was treated with a
restriction enzyme Dra I. The Dra I/Hind III-truncated DNA fragment
25 thus obtained was isolated by electroelution. Separately, from the vector
pBluescript II SK (Stratagene) in which a DNA fragment including the
promoter site, exon 1, intron 1 and exon 2 of a goat β casein gene was
subcloned, a Stu I/Dra I-truncated DNA fragment including the promoter
and a partial exon 1 extending to one nucleotide on the upstream side of
30 the translation start codon, was obtained by enzymatic cleavage and by
isolation on agarose gel. The two DNA fragments were inserted together
in a pRC/RSV vector which was opened by the double-digestion with
restriction enzymes Nru I and Hind III, to make a novel plasmid pGbc_L.
This procedure is summarized in Fig. 4.

3) Identification of pGbc_S and pGbc_L Constructs

35 The successful construction of plasmids pGbc_S and pGbc_L was

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confirmed by base sequencing analysis. The base sequencing analysis was performed by using a Sequenase kit Ver 2.0, provided by Amersham U.S.A, according to the protocol of the provider. For the sequencing, two primers V1 and V2 were designed to have a base sequence of 5'-AGG
5 CAA GGC TTG ACC GAC-3' and 5'-GGA GGG GCA AAC AAC AGA
TG-3', respectively.

EXAMPLE II: Recombination between hGCSF Gene and Mammalian Expression Vector for HC11 Cell

10 An hGCSF gene was inserted in each of the vectors pGbc_S and pGbc_L according to the recombination strategy illustrated in Figs. 5 and 7. In the recombination, the β -casein exon 1 was directly linked to the exon 1 of the hG-CSF gene while the translation start codon for the exon 1 of the hG-CSF gene was kept available.

15 In more detail, first, the pUC 19 vector in which an hG-CSF gene was subcloned, was digested with BamH I and Xba I and electrophoresed on an agarose gel to separate the resulting two DNA fragments from each other. These two DNA fragments, which consisted of Fragment 1 and the other fragment, were separately purified using a GeneClean II kit, commercially available from BIO101. Fragment 1 was believed to
20 comprise exon 2 to poly A signal. The other fragment comprising the vector and exon 1 was cut with a restriction enzyme Pst I and then, the smaller fragments cut were run on an agarose gel to separate them from each other. Purification using a GeneClean II kit, commercially available from BIO101 afforded Fragment 2 which comprised one nucleotide on
25 the 5' side of the translation start codon through one nucleotide on the 5' side of the exon 2. Fragment 1 and Fragment 2 together were inserted in a plasmid pBluescript II SK by ligation to give a novel recombinant plasmid pBluescript II SK-hGCSF. This plasmid was double-cut with restriction enzymes Hind III and Xba I to obtain a modified hG-CSF gene
30 fragment which was, then, inserted in a pGbc_S vector which was previously opened by digestion with Hind III and Xba I. In result, a novel recombinant plasmid pGbc_S-hGCSF was obtained.

35 Separately, a pGbc_L vector was opened by enzymatic digestion with Hind III and Xba I. After being treated with an alkali phosphatase and electrophoresed on an agarose gel, the opened pGbc_L vector was

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purified using a Geneclean II kit. A DNA fragment obtained by the endonuclease digestion of a goat β -casein promoter and a modified hGCSF gene fragment obtained by double digestion of the pBluescript II SK-hGCSF with Hind III and Xba I are together ligated to the opened
5 vector, to give a novel recombinant plasmid pGbc_L-hGCSF.

The successful construction of the novel recombinant plasmids pGbc_S-hGCSF and pGbc_L-hGCSF was confirmed by base sequencing analysis. The base sequencing analysis was performed by using a Sequenase kit Ver 2.0, provided by Amersham U.S.A, according to the
10 protocol of the provider. For the sequencing, three primers V1, V2 and P1 were designed to have a base sequence of 5'-AGG CAA GGC TTG ACC GAC-3', 5'-GGA GGG GCA AAC AAC AGA TG-3', and 5'-CAC TAT TGG TTT TAT TTC-3', respectively.

EXAMPLE III: Recombination between hGMCSF Gene and Mammalian
15 Expression Vectors pGbc_S and pGbc_L for HC11 Cells

The recombination was carried out by following the strategy illustrated in Figs. 6 and 8.

First, the hGMCSF gene which was subcloned in a pUC19 vector, was extracted by digestion with restriction enzymes BamH I and EcoR I
20 and by purification with a Geneclean II kit (BIO101) from an agarose gel on which the digested DNA fragments were electrophoresed. Then, the hGMCSF gene was inserted by ligation to a pBluescript II SK vector (Stratagene) which had been digested with the same endonucleases and then, with a bovine alkali phosphatase, to afford a recombinant plasmid
25 pBluescript II SK-hGMCSF. A modified gene was recovered from this recombinant plasmid by digestion with Hind III and Xba I, electrophoresis on an agarose gel and purification with a Geneclean II kit (BIO101) and then, ligated to a pGbc_S vector which were previously treated with the same endonuclease and then with a bovine alkali
30 phosphatase, to construct a novel plasmid pGbc_S-hGMCSF.

Similarly, a novel plasmid pGbc_L-hGMCSF was constructed by ligating a DNA fragment obtained by the endonuclease digestion of a goat β -casein promoter and a modified hGMCSF gene fragment obtained by the double digestion of the pBluescript II SK-hGMCSF with Hind III and

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Xba I to an opened pGbc_L vector which was obtained by the treatment with restriction enzymes Hind III and Xba I and then with a bovine alkali phosphatase and by the purification from an agarose gel on which electrophoresis was done.

- 5 The successful construction of the expression vectors is confirmed through base sequencing analysis using the primers VI, V2 and P1.

EXAMPLE IV: Construction of the Vector for Transfection

- 10 Mammalian expression vectors for tranfection with the same gene structure as that of Fig. 1, were constructed according to the recombination strategy of Fig. 9. They could be prepared by inserting a goat β -casein promoter site, a physiological activating substance gene and a bovine growth hormone terminator in a pBluescript II SK vector (Stratagene).

- 15 In more detail, from a goat β -casein gene or its fragment, a DNA fragment including the promoter site and a partial exon 1 extending to one nucleotide on the upstream side of the translation start codon, was obtained by cutting with Sac I and Hind III. After a purification procedure comprising the steps of extracting with a phenol:chloroform 1:1 solution, precipitating with 95% ethanol, and dissolving in distilled water, 20 the DNA fragment was further cut with a restriction enzyme Dra I. Electrophoresis on an agarose gel and purification by use of a GeneClean II kit (BIO101) afforded Fragment 1, which comprised the partial exon 1.

- 25 Separately, a stretch of DNA which covered from 501 nucleotides to one nucleotide on the 5' side of the translation start codon for the exon 1 of the β -casein gene, was obtained by a PCR using the primers CAS-F1 and CAS-R1 and, then, cleaved by Dra I and Hind III. Fragment 2, which comprised the exon 1, was obtained by electroelution.

- 30 Fragments 1 and 2 were inserted by ligation in a pBluescript II SK vector (Stratagene) which had been enzymatically treated with Sac I and Hind III and then, with a bovine alkali phosphatase. The recombinant plasmid thus obtained was opened by double-digestion with Hind III and EcoR I. To this opened cloning site, a DNA fragment in which a physiological activating substance gene was linked to a bovine growth 35 hormone terminator, was inserted.

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The success of the above recombination procedure was confirmed through a base sequencing analysis using the primer P1.

EXAMPLE V: Expression of hGMCSF in Mouse Mammary Gland-derived HC11 Cells

5 The plasmids pGbc_S-hGMCSF and pGbc_L-hGMCSF which resulted from the recombination between the plasmid pGbc_S and the hGMCSF gene and between the plasmid pGbc_L and the hGMCSF gene, respectively, in Example III, were purified using QIAGEN-tip 100 (Qiagen) before being introduced into HC11 cells.

10 After being transformed with each of the plasmids, E.coli cells were inoculated in 150 ml of LB medium containing ampicillin at a concentration of 100 μ g/ml and incubated at 37 °C for 10 hours with agitation. The cells were harvested by centrifugation, and the plasmids were purified from the cells using the protocol recommended by Qiagen.

15 Transfection of the purified plasmids into HC11 cells was accomplished by using an electroporator, commercially available from Invitrogen. HC11 cells were densely grown on T75 flasks for tissue culture in a 5% CO₂, 37 °C incubator until they covered 80% of the bottom area of the flask. Thereafter, they were floated with a trypsin solution (Gibco BRL) and suspended with a PBS buffer. This cell suspension was centrifuged at 1,500 rpm to harvest the cells which was, then, washed with a chilled PBS buffer. A small volume of the cell solution was taken to count cell number and the remaining volume was precipitated by centrifugation at 1,500 rpm. The precipitated mass of the cells was diluted with a PBS buffer to give a cell suspension having 3x10⁶-1x10⁷ cells/500 μ l.

20 20 μ g of the purified plasmid and 500 μ l of the cell suspension were added in a 0.4 cm cuvette which was, then, put in ice for 10 min. After being set to any one of any one of 71, 250, 500 and 1000 μ F, the electroporator was controlled in voltage and resistances. The electroporator was charged for 3 min in order to apply pulses to the cuvette which was brought into the chamber of the electroporator. Then, the cuvette was put in ice for 10 min, after which the cell suspension in the cuvette was added with 1 ml of a growth medium and poured on 4 ml of a growth medium in a T25 flask. After culturing for 24-48 hours in a

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5% CO₂, 37 °C incubator, the cells were provided a fresh medium RPMI 1640 (Gibco BRL), supplemented with fetal bovine serum at a final concentration of 10%, epidermal growth factor at 10 ng/ml, insulin at 5 µg/ml, and antibiotics gentamicin at 50 µg/ml and geneticin (Sigma) at 200 µg/ml, to select the transfected cells. The selective medium was refreshed every second or third day. At 7 days after culturing in the selective medium, only the cells into which the plasmids were introduced, survived. These transfected cells continued to grow densely.

After the selection, the selective medium was changed with an induction medium comprising RPMI 1640 media (Gibco BRL) supplemented with insulin at a final concentration of 5 µg/ml, geneticin (Sigma) at 200 µg/ml, gentamicin 50 µg/ml, goat prolactin at 5 µg/ml and dexamethasone at 1 µM. The cells were cultured in a 5% CO₂, 37 °C incubator for 4 days without refreshing the medium.

The hGMCSF produced as a result of the expression induction of the gene, was secreted into the medium. A Western blotting technique was used for the qualitative analysis of the secreted product while an enzyme linked immunosorbent assay (ELISA) was for a quantitative analysis. As a primary antibody for the Western blotting, an anti-human GM-CSF mouse Ig G was used for the analysis of the expression of human granulocyte macrophage colony stimulating factor. A horse radish peroxidase-conjugated anti-mouse Ig G was used as a secondary antibody for the Western blotting. For ELISA, anti-human GM-CSF goat Ig G polyclonal antibodies were first attached on 96 well plates which were, then, treated with the expressed product as a corresponding antigen or with a commercially available factor used as a standard. To these were linked the anti-human GM-CSF monoclonal antibodies which were the same as used in the Western blotting. The resulting antibody complexes were treated with alkali phosphatase-conjugated anti-mouse Ig G monoclonal antibody with the aim of inducing a coloring reaction (Ed Harlow and Davis Lane (1989) *Antibodies A Laboratory Manual*, Cold Spring harbor Laboratory Press, New York).

The data obtained from the Western blot and ELISA are given in Figs. 10 and 11 and show that the novel recombinant vectors pGbc_S and pGbc_L both induce the protein to be expressed at a level of 40 ng/ml.

EXAMPLE VI: Expression of hGCSF in HC11 cells

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The plasmids pGbc_S-hGCSF and pGbc_L-hGCSF which resulted from the recombination between the plasmid pGbc_S and the hGCSF gene and between the plasmid pGbc_L and the hGCSF gene, respectively, were purified using QIAGEN-tip 100 (Qiagen) in the same manner as that of Example V before being introduced into HC11 cells. The introduction was achieved by a calcium phosphate coprecipitation method or an electroporation method. Following transfection, culture in a selective medium comprising the same composition as that of Example V left geneticin-resistant colonies only. These screened colonies were transferred onto T75 flasks and grown densely thereon. The cells were provided with a fresh induction medium containing lactating hormones, prolactin and dexamethasone, and cultured for 4 days, after which the medium and the cells were separated by centrifugation. The supernatant was utilized for Western blotting analysis and ELISA. The data obtained are given in Fig. 12, showing that the novel recombinant vectors pGbc_S and pGbc_L both induce the protein to be expressed at a level of 40 ng/ml.

EXAMPLE VII: Expression of hGMCSF in Transgenic Mice

The novel recombinant vector pGbc-hGCSF which resulted from the recombination between the vector pGbc and the hGMCSF gene, was purified with the aid of QIAGEN tip 100 and digested with restriction enzymes BssH I and Kpn I, followed by extraction with a GeneClean II kit (BIO101) from an agarose gel. For use in microinjection, the extracted vectors were further purified by following the protocol recommended by Schleicher & Schuell and then, dialyzed in a solution comprising 10 mM Tris-Cl (pH 7.2) and 10 mM EDTA, to give a DNA solution at a concentration of 40 ng /ml.

Into the male pronuclear site of a CBA line mouse oosperm, the finally purified expression vector was introduced by microinjection. This oosperm was nidated in the womb of a surrogate mother. From the tails of the progenies of the surrogate mother, genomic DNAs were isolated. Which mouse was transgenic was identified by PCR, as shown in Fig. 13, and confirmed with a Southern blotting analysis.

The progeny mice which were confirmed to have the gene introduced into their genomic DNAs were let to couple non-transgenic,

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normal mice to produce next generation progenies. 10 days after birth, the transgenic parturient mice were segregated from their offspring for 3 hours. Following peritoneal injection of oxytocin along with an anesthetic, milk was extracted from the parturient mice.

5 The expression level of the genes was assayed in the same manner as in cell level, using Western blotting and ELISA for qualitative and quantitative analyses, respectively. The antibodies used in these analyses were the same as those suggested in Example V. The results are given in Figs. 14 and 15. From the Western blot, it is apparent that a protein from
10 the transgenic mice is the same as a commercially available hGCSF (Gibco BRL) and is expressed through the mammary gland tissue of the transgenic mice, as shown in Fig. 14. The data of the ELISA show that the hGCSF is expressed at a level of 150 ng/ μ l, as shown in Fig. 15.

15 EXAMPLE VIII: Expression of hGMCSF in Transgenic Mice

 The novel recombinant vector pGbc-hGMCSF which resulted from the recombination between the vector pGbc and the hGMCSF gene, was purified with the aid of QIAGEN tip 100 and digested with restriction
20 enzymes BssH I and Kpn I, followed by extraction with a GeneClean II kit (BIO101) from an agarose gel. For use in microinjection, the extracted vectors were further purified by following the protocol recommended by Schleicher & Schuell and then, dialyzed in a solution comprising 10 mM Tris-Cl (pH 7.2) and 10 mM EDTA, to give a DNA
25 solution at a concentration of 40 ng/ml.

 Into the male pronuclear site of a CBA line mouse oosperm, the finally purified expression vector was introduced by microinjection. This oosperm was nidated in the womb of a surrogate mother. From the tails of the progenies of the surrogate mother, genomic DNAs were isolated. Which mouse was transgenic was identified by PCR, followed by
30 confirmation with a Southern blotting analysis.

 Milk was taken from the transgenic mice and subjected to Western blotting and ELISA. The data of the Western blotting analysis in which a commercial hGMCSF (BIO101) was used as a positive control, shows that a protein from the transgenic mice is hGCSF and is expressed
35 through its mammary gland tissue. From the ELISA, the hGCSF from the transgenic mice was revealed to be expressed at a level of 130 ng/ μ l, as

shown in Fig. 16.

Industrial Applicability

As described hereinbefore, the mammary gland tissue-specific expression systems using the β -casein promoter site of Korean native goats, in accordance with the present invention, enable physiological activating substances to be produced in vivo, that is, in mammary gland tissue-derived cells as well as in transgenic animals. Therefore, the proteins obtained are those which experience the posttranslational modification and thus can maintain their normal activity in the human body. Also, the expression systems according to the present invention make it possible to easily produce the proteins at a great amount. Where the proteins are produced in a cell level, a lactating hormone is used as a potent expression inducer. In the case of transgenic animals, the proteins can be easily obtained through the milk secreted therefrom. Thus, the scale-up of the production of the proteins to the extent of industrialization can be achieved. Additionally, the mammary gland tissue-specific expression systems according to the present invention are very advantageous in isolating and purifying the desired protein with ease and safety.

The following are the great industrial advantages which the mammary gland tissue-specific expression system of the present invention will bring about.

First, because the target proteins which are produced in the mammary gland tissue-specific expression systems which employ the β -casein promoter site of Korean native goats, experience the same posttranslational modification as that which the corresponding naturally occurring proteins do, the target proteins can sustain their activity in the human body. Overcoming the limitation the conventional expression systems using E.coli as a host has, the expression systems of the present invention can be equally applied to all kinds of the physiological activating substances.

Second, the goat β -casein promoter used in the present invention enable the accompanied structural gene to be inducibly expressed at a great amount, leading to a great decrease in the production cost of corresponding proteins. It is well known that the expression of the goat

β casein gene can be induced in a highly level by the hormones from the mammary gland tissue, that is, lactating hormones. The mammary gland tissue-derived cells transfected with the expression systems employing the goat β -casein promoter can produce the desired proteins at an amount of 40 ng/ml by the treatment with a trace amount of an inducer, a lactating hormone, while the transgenic animals with the expression systems can produce the desired proteins at an amount of 130-150 ng/ μ l by the animals' own hormones. To our knowledge, greater amounts of these products are possible to obtain by modifying the length of the goat β -casein promoter site and expression conditions. Therefore, the present invention suggests a novel mass-production method for proteins.

Thirdly, the scale-up for the mass-production of target proteins can be easily achieved through the mammary gland tissue-specific expression systems of the present invention without requiring a significant cost. Because the target proteins are secreted in milk, the scale-up can be achieved simply by increasing the number of the transgenic animals. This can save the cost further required to scale up protein production from a laboratory level to an industrial level. That is, the production cost of the physiological activating substances can be significantly reduced by use of the mammary gland tissue-specific expression systems of the present invention.

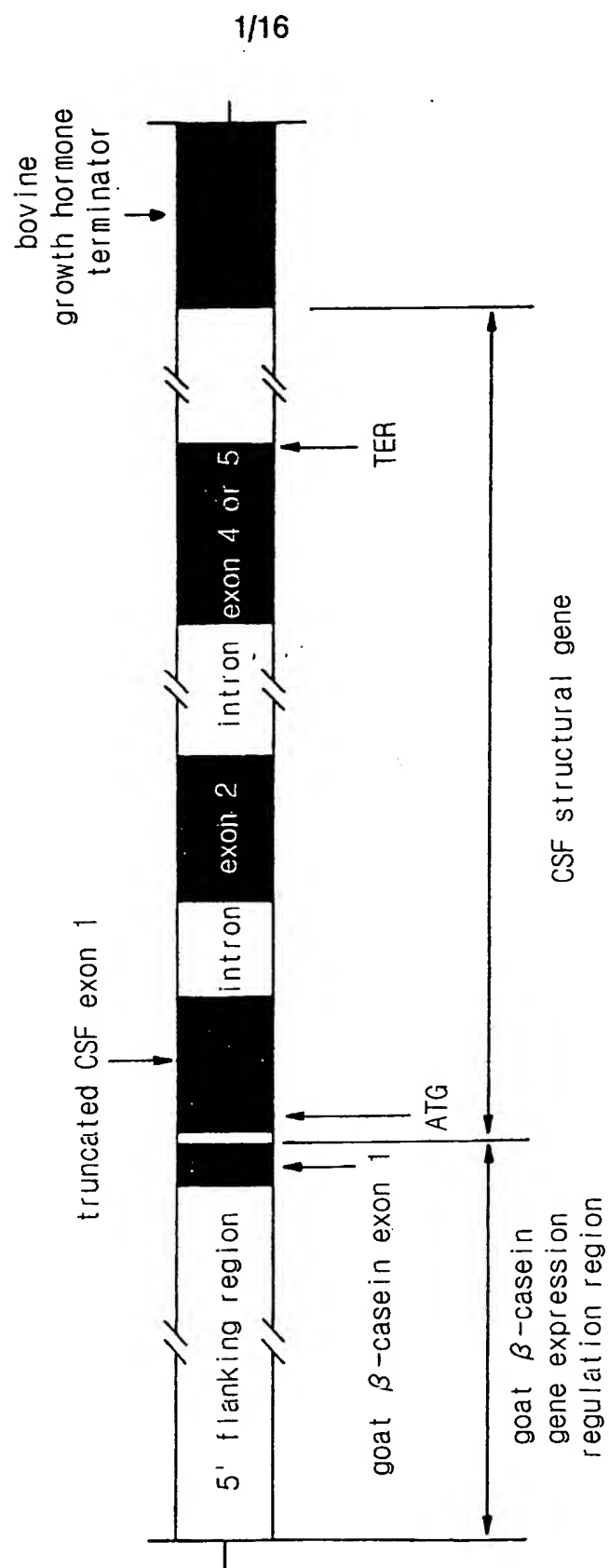
Fourth, the present invention is based on the simpleness and harmlessness of the proteins secreted from the mammary gland. There are a few kinds of proteins which are expressed in mammary gland tissues, so that the proteins each can be isolated by ordinary techniques. Further, no harmful proteins are detected from milk.

The present invention has been described in an illustrative manner, and it is to be understood the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. For example, other various physiological activating substances can be expressed through the expression systems of the invention with a little aid of well-known DNA recombination techniques. Therefore, the present invention should not be confined only to the expression of hG-CSF or hGM-CSF, which is illustrated through the specification. It is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Claims

1. A recombinant vector pGbc (KCTC 0515BP), using a β -casein promoter site of Korean native goats.
- 5 2. A recombinant vector pGbc_L (KCTC 0514BP), using a β -casein promoter site of Korean native goats.
3. A recombinant vector pGbc_S (KCTC 0513BP), using a β -casein promoter site of Korean native goats.
- 10 4. A method for producing physiological activating substances, in which the physiological activating substances are expressed using any one of the vectors pGbc, pGbc_L and pGbc_S as claimed in claims 1 to 3.
5. A method as set forth in claim 4, wherein the physiological activating substances are expressed in mammary gland tissue-derived cells which are transfected with the pGbc_L vector or the pGbc_S vector.
- 15 6. A method as set forth in claim 4, wherein the physiological activating substances are expressed in transgenic animals with the pGbc vector.
7. A method as set forth in claim 5, wherein said mammary gland tissue-derived cells are of HC11 line.
- 20 8. A method as set forth in claim 7, wherein the HC11 cells are transfected with a gene-carrying pGbc_L vector or pGbc_S vector by calcium phosphate coprecipitation or electroporation.
9. A method as set forth in claim 6, wherein the transgenic animals are made by microinjection of a gene-carrying pGbc vector into the male pronucleus of the animals.

Fig.1



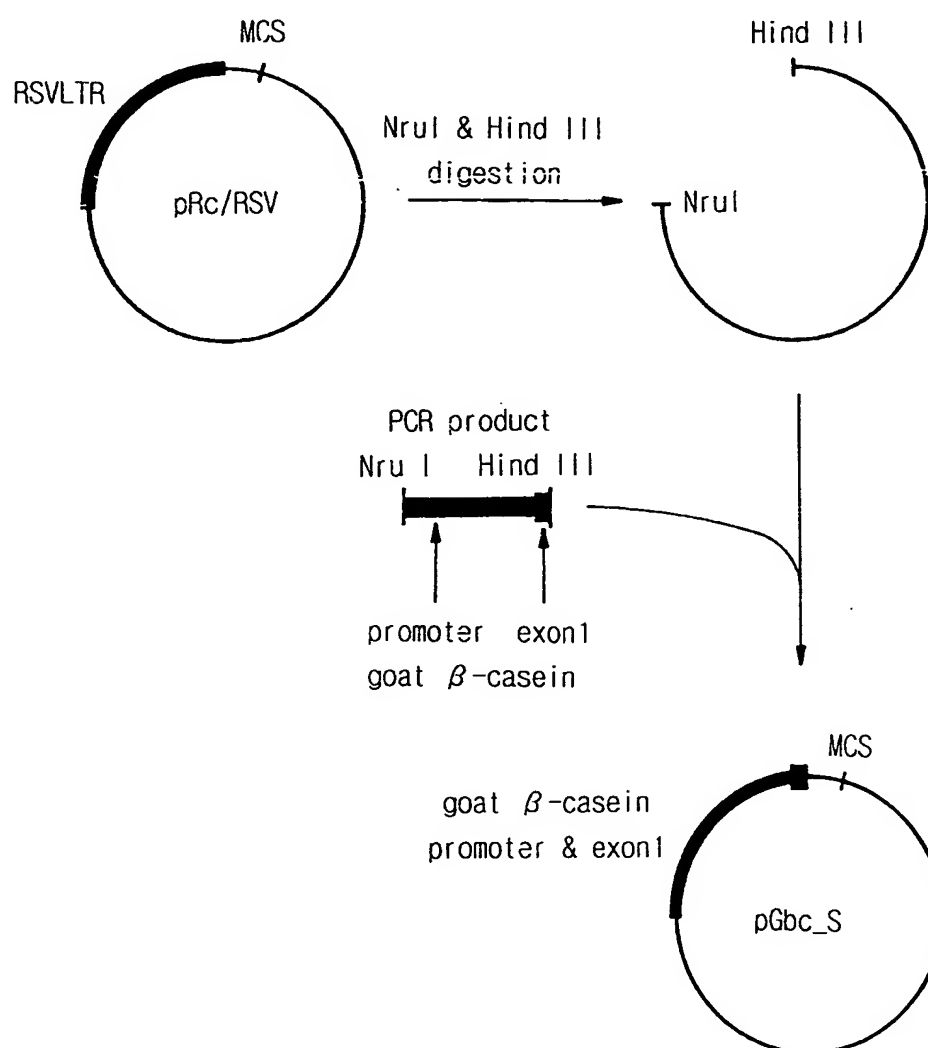
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Fig.2

1 aagcttcttt ctttagtata ttgtaagga tttcttgatc aagattttac ctacttttct
61 ggtccaattg gtgagagaca gtcataagga aatgctgtgt ttattgcaca ataigtaaag
121 catcttcctg agaaaataaa agggaaatgt tgaatgggaa ggatatgctt tcttttgtat
181 tccttttctg agaaatcaga ctttttcacc tgtggccttg gcacaaaagc taacaaataa
241 aggcataatga agtagccaag gccttttcta gtatatctat gacactgagt tcatttcac
301 atttattttc ctgacttcct cctgggtcca tatgagcagt cttagaatga atattagctg
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721 gactctattg tagtacttag ggtaataccc tcctcctgta tgggctttca ttttcttct
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841 atgaggccaa acttgagctg tcccatitta ataaatctgt ataataatat tgttctacaa
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1021 cccttttctt tttctcttc tcacctcat ctactcctt ttccttgcaa tcatgacct
1081 agattcactg tttgatttg cttgcattg tgtgtgctga gttgcgtctg actgttatca
1141 accccatgaa tgatagcca ccaggctcta ctgtccatga aattttccag tcaagaatac
1201 tggagtggat tgcatttcct actccattg attaatatg tgactttfaa atttctttt
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1381 tgttcagggt ggcatgaaat tgtgttctt gtgtggcctg tatatttctg ttgtgtatta
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1501 ataatttgg gactgggcca aaacttccgt gcatcccagc caagatctgt agctactgga
1561 caatttcatt tcctttatca gatgtgagt tattcctgtt aaaatgctcc ccagaattc
1621 tggggacaga aaaataggaa gaattcattt cctaatactg cagatttcta ggaattcaaa
1681 tccactgttg gttttattc aaaccacaaa attagcatgc cattaaatc tatatataaa
1741 cagccactaa atcagatcat tatccattca gcttctcctt cacttcttct cctctactt
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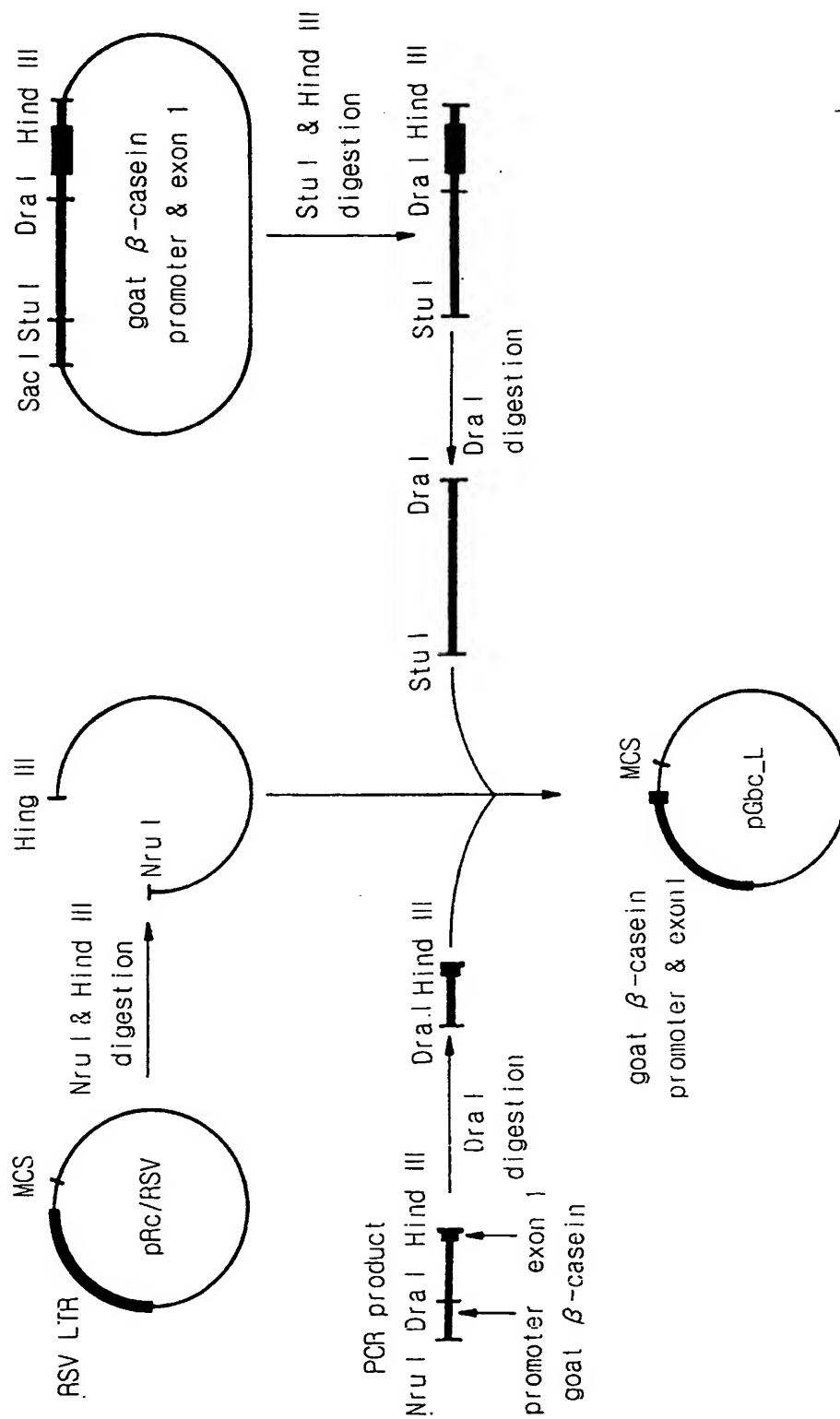
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Fig.3



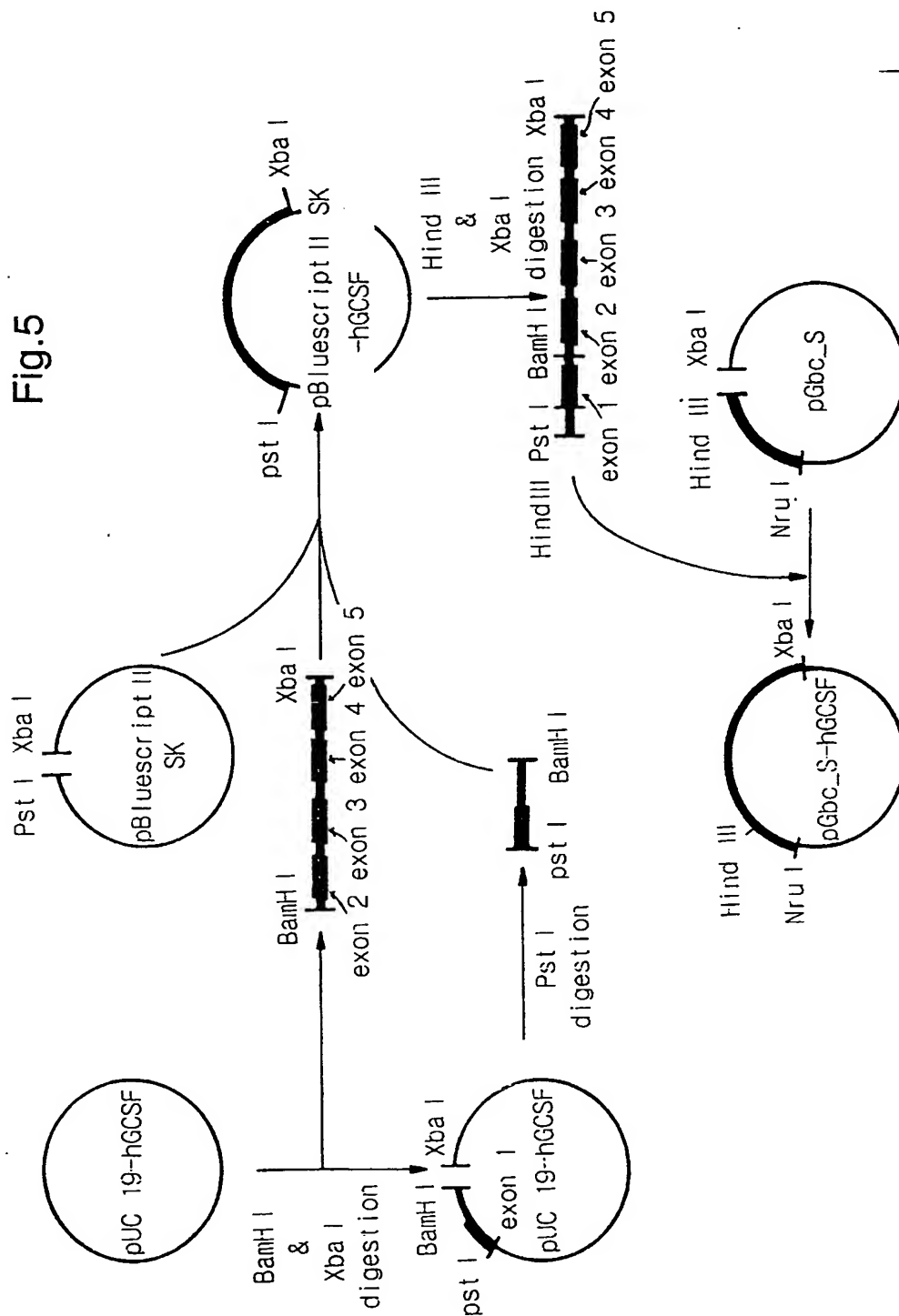
4/16

Fig.4



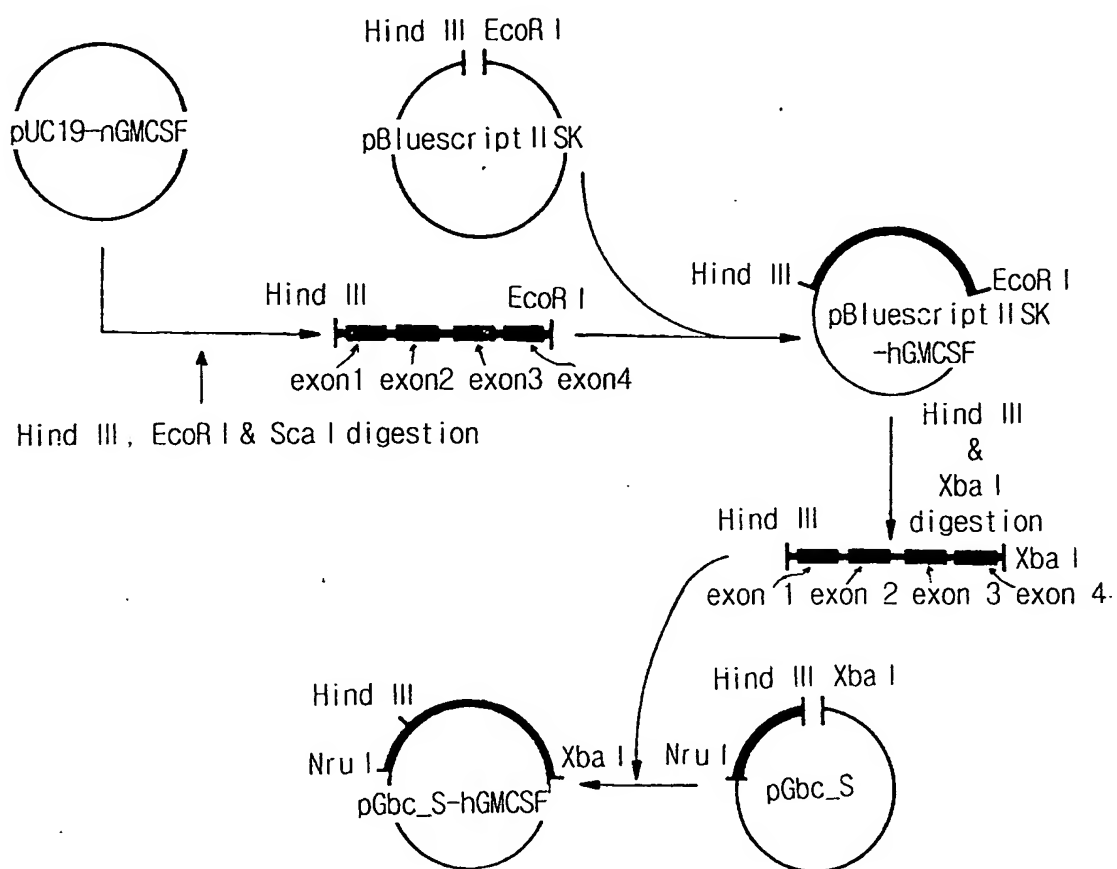
5/16

Fig.5



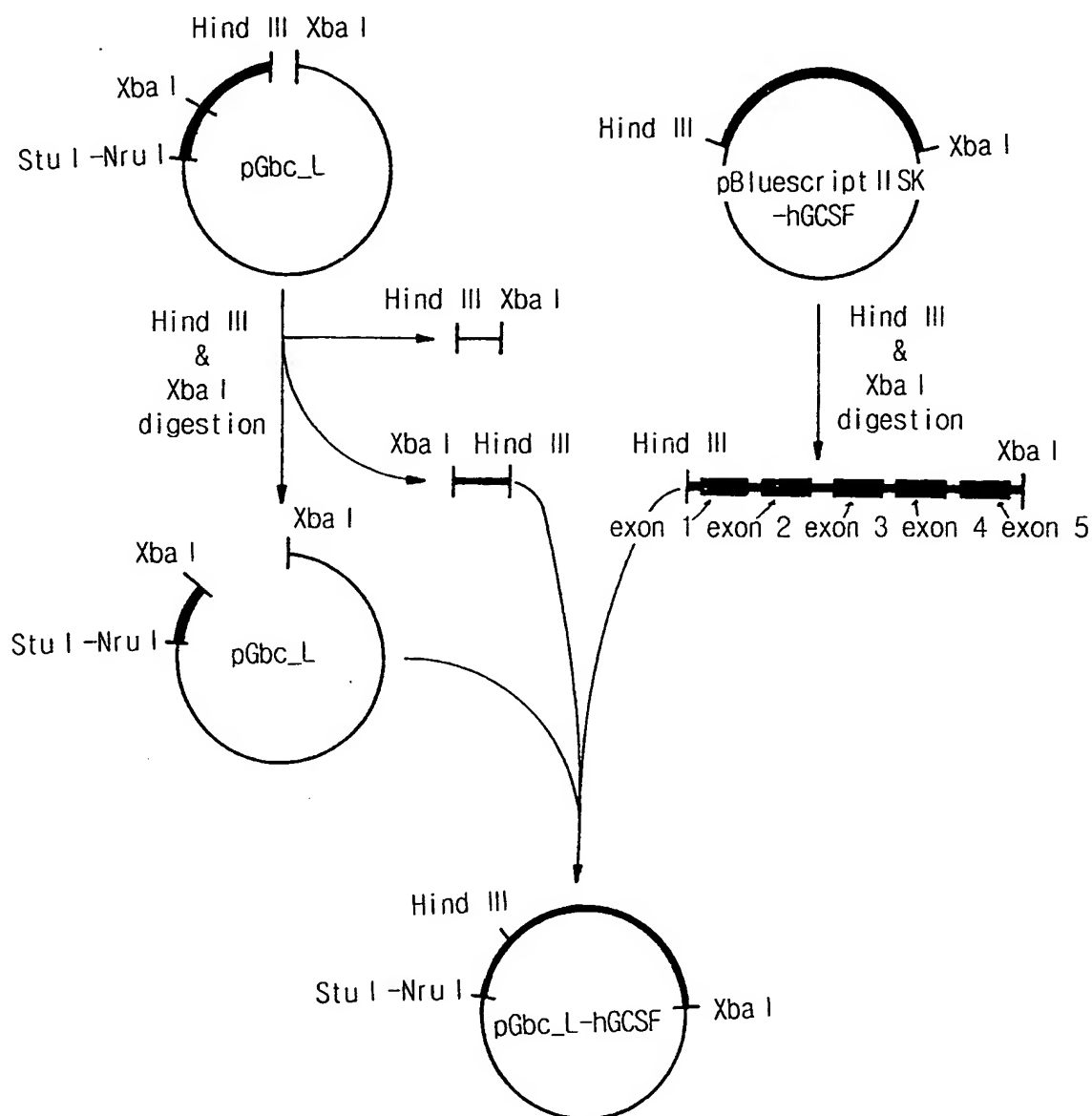
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Fig.6



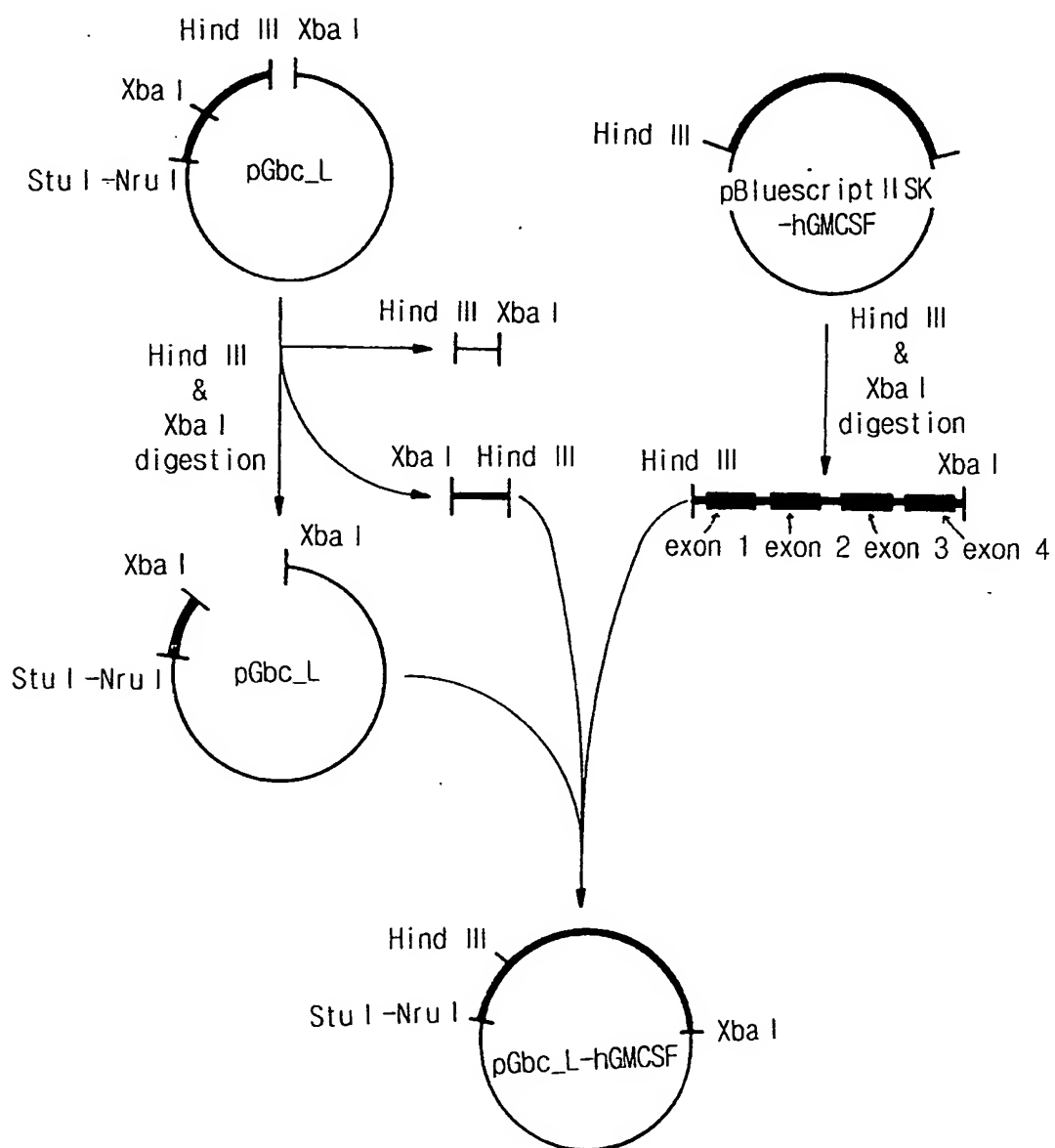
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Fig.7



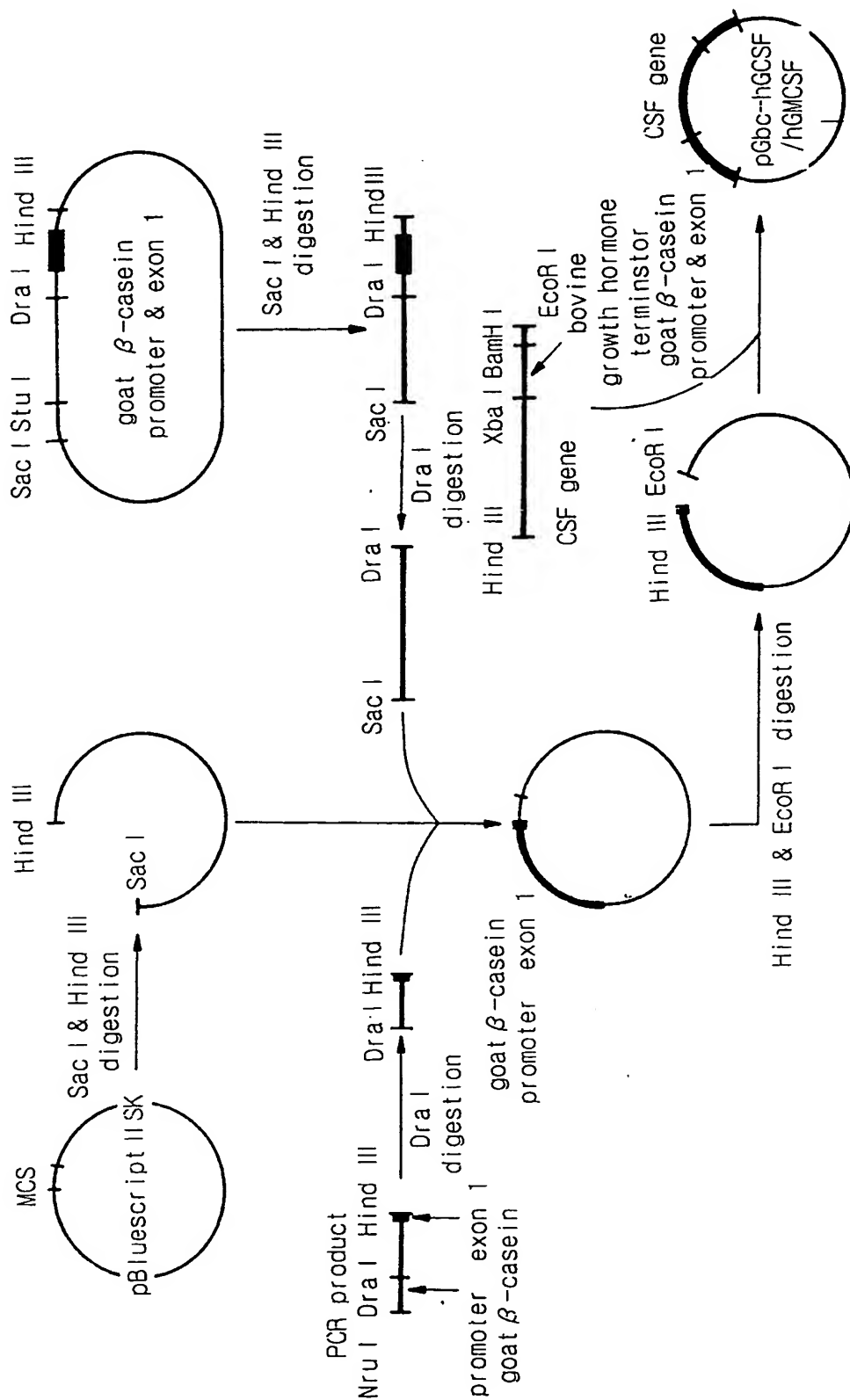
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Fig.8



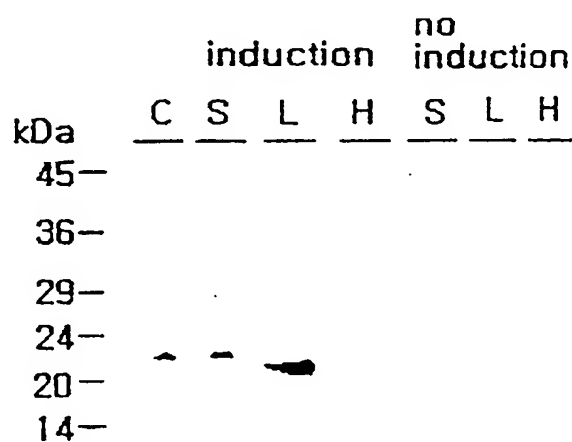
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Fig.9



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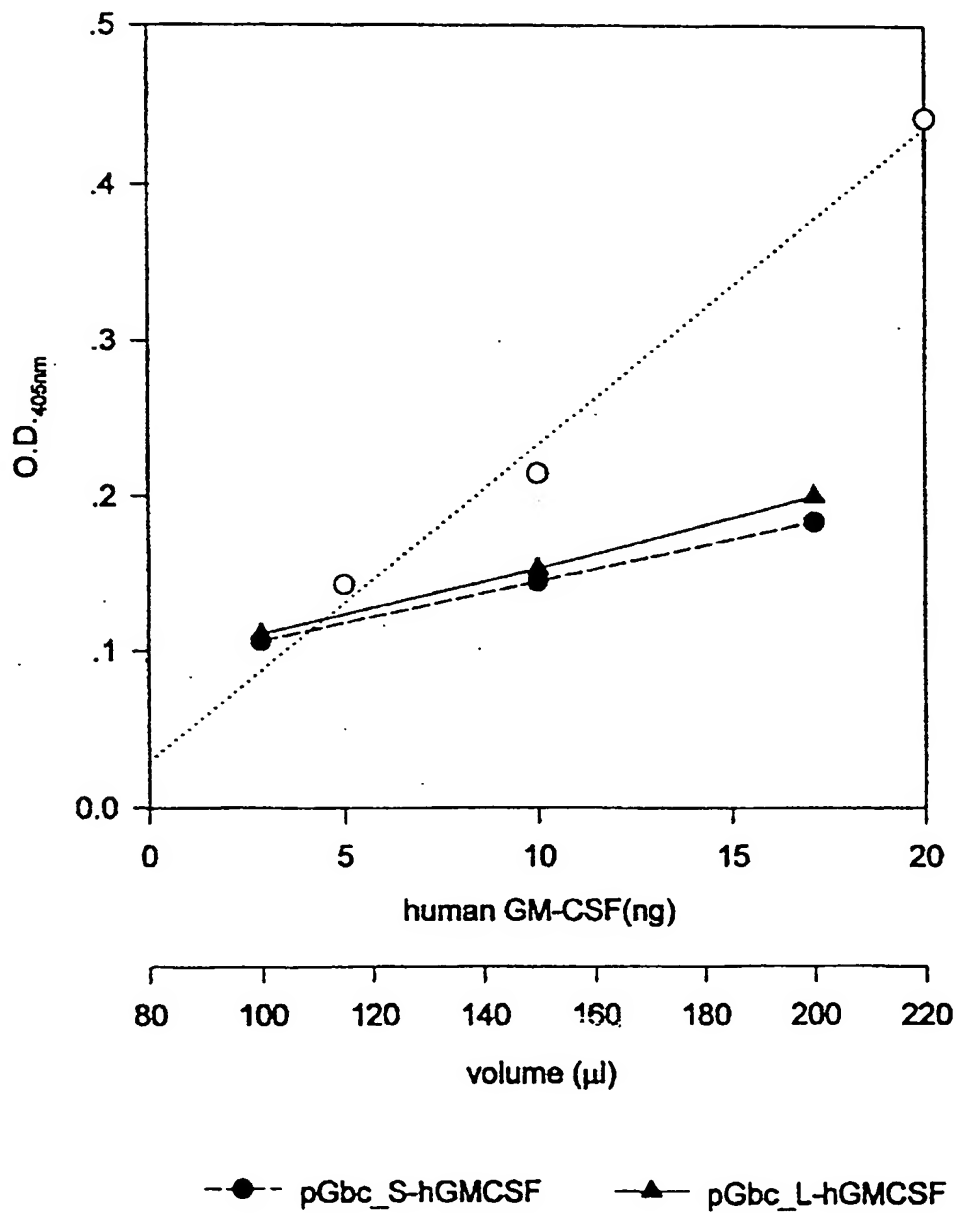
Fig.10



C : commercial hGM-CSF
S : pGbc_S-hGMCSF transfected
L : pGbc_L-hGMCSF transfected
H : HC11 cell , not transfected

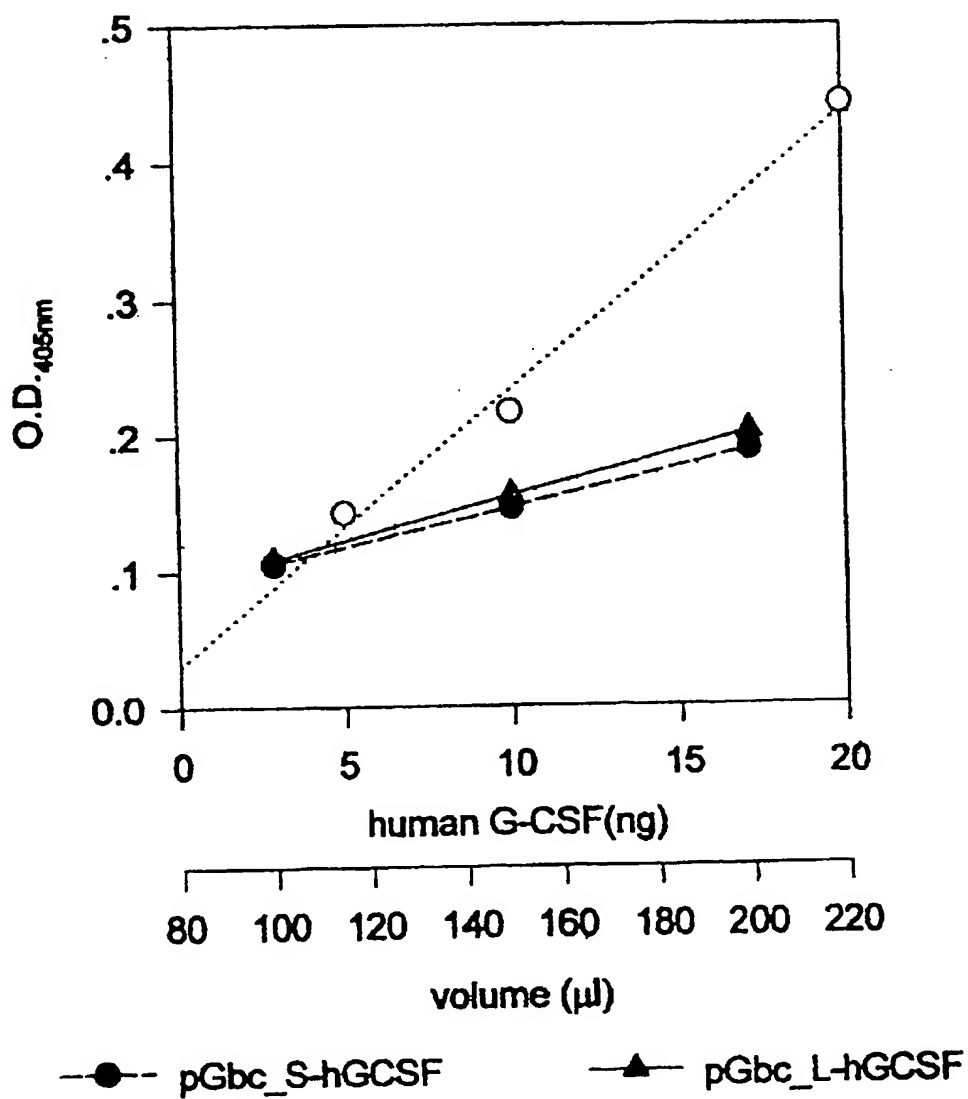
11/16

Fig.11



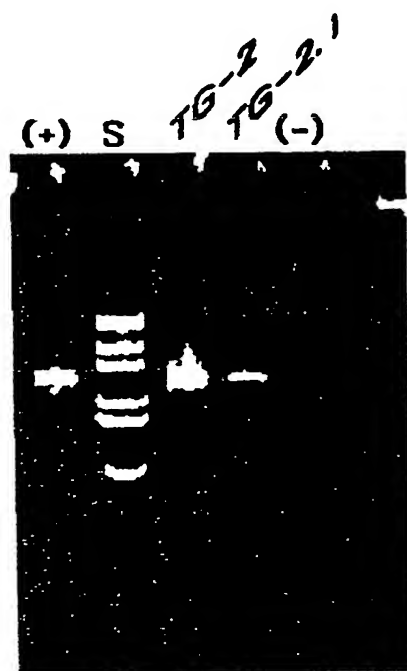
12/16

Fig.12



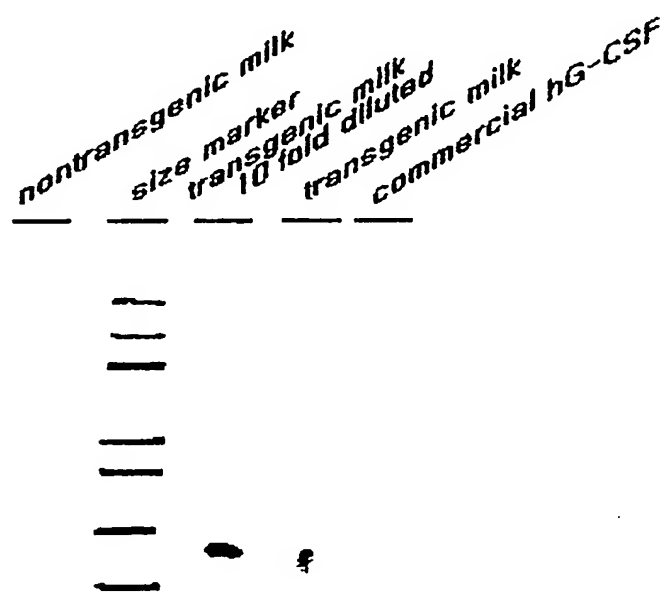
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Fig.13



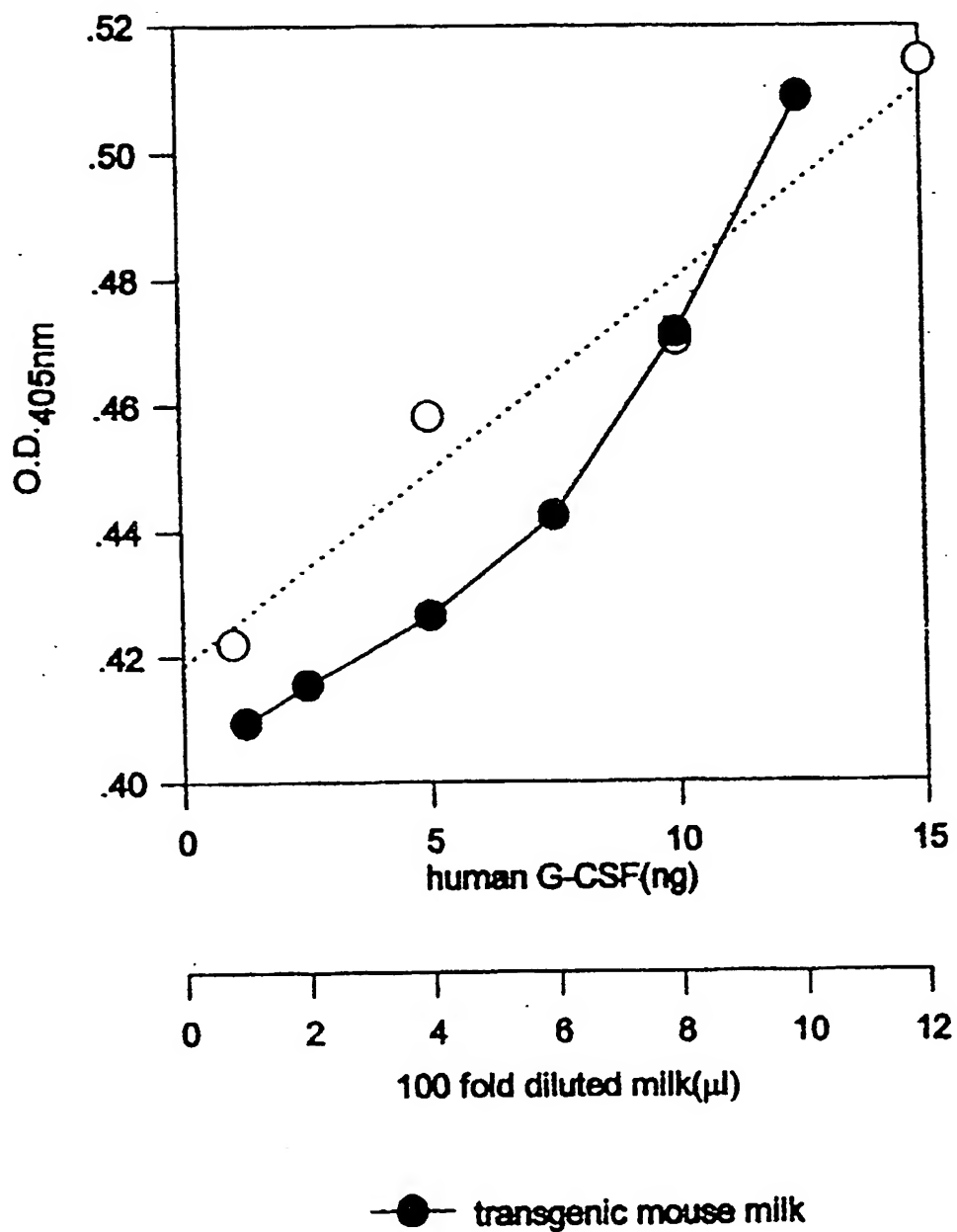
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Fig.14



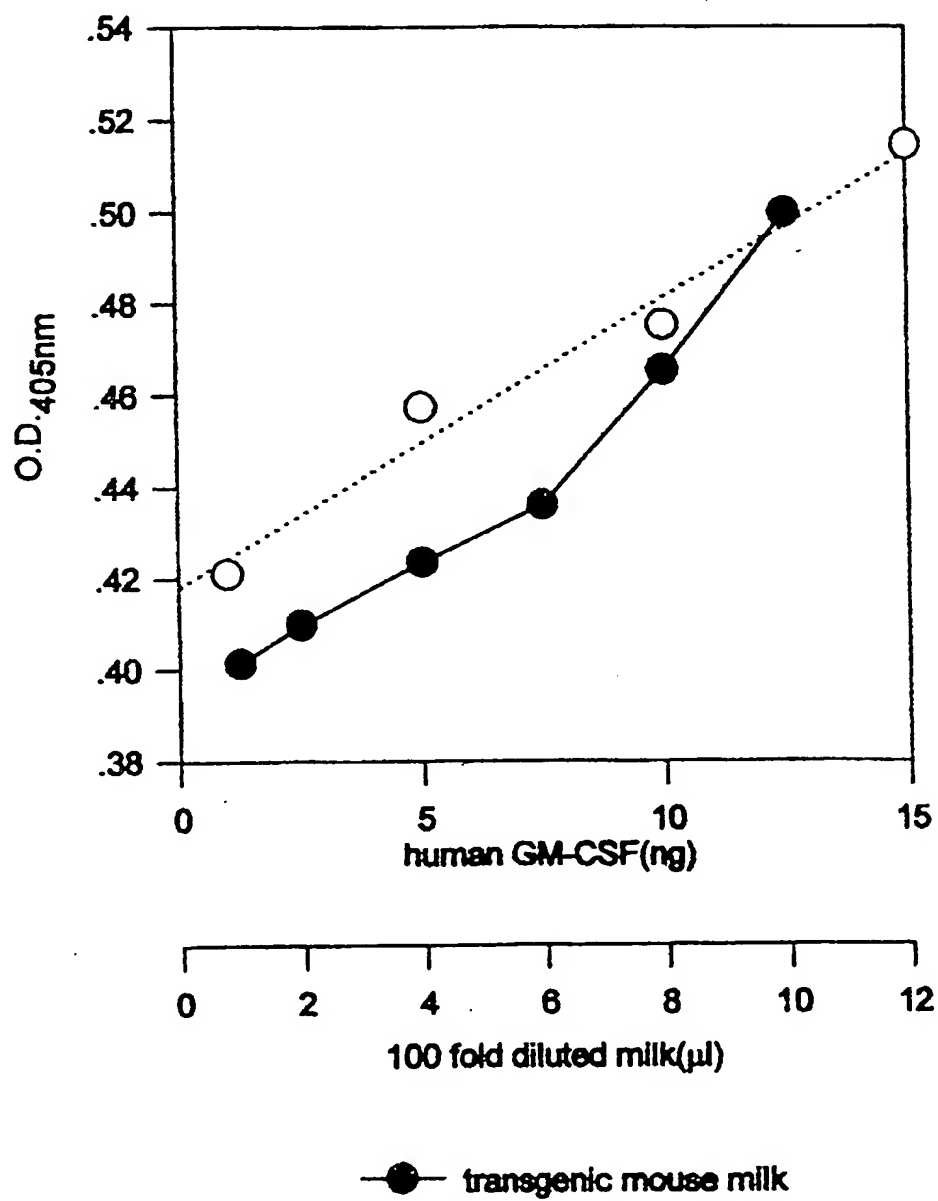
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Fig.15



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Fig.16



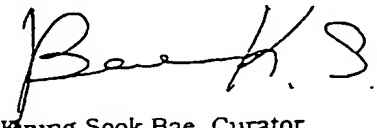
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Choi, Duk-In
Korea Advanced Institute of Science & Technology
#373-1, Kusong-dong, Yusong-ku, Taejon 305-701,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: E. coli JM83/pGbc-hGCSF	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0515BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on March 10 1997 and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on August 17 1998 .	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures Address: KCTC, KRIBB #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Kyung Sook Bae, Curator Date: August 20 1998

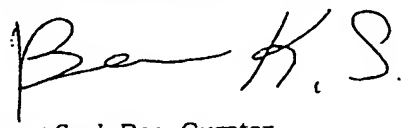
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#373-1, Kusong-dong, Yusong-ku, Taejon 305-701.
Republic of Korea

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Identification reference given by the DEPOSITOR: <i>E. coli</i> JM83/pGbc-L	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0514BP
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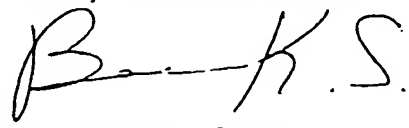
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Republic of Korea

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Identification reference given by the DEPOSITOR: <i>E. coli</i> JM83/pGbc-S	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0513BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00277

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 N 15/63, 15/12, 15/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 N 15/63, 15/12, 15/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95/17 085 A1 (GENZYME TRANSGENICS CORPORATION) 29 June 1995 (29.06.95), claims 1-3,8-10.	1-3
A	Patent Abstracts of Japan, Vol. 97, No. 6, 1997, JP 9-037 786 A (TERUMO CORP.) 30 June 1997 (30.06.97).	1-3

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

07 June 1999 (07.06.99)

Date of mailing of the international search report

15 June 1999 (15.06.99)

Name and mailing address of the ISA/AT

Austrian Patent Office
Kohlmarkt 8-10; A-1014 Vienna
Facsimile No. 1/53424/535

Authorized officer

Wolf

Telephone No. 1/53424/436

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 98/00277

In Recherchenbericht angeführtes Patentedokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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		AU B2 688845	19-03-1998
		AU A1 73079/98	20-08-1998
		CA AA 2178941	29-06-1995
		EP A1 741515	13-11-1996
		EP A4 741515	27-05-1998
		JP T2 9506779	08-07-1997
		NZ A 278744	24-04-1997
		US A 5827690	27-10-1998
		US A 5849992	15-12-1998